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Sir:

RULE 132 DECLARATION OF DAVID A. SLEPER

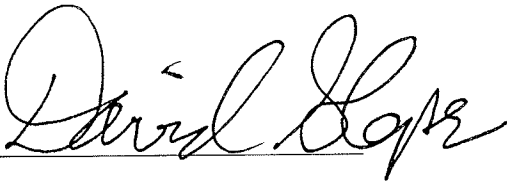
1. This Declaration is being submitted to present certain information for the Examiner's review. This information traverses obviousness rejections found in the Office Action dated October 18, 2006.
2. My name is David A. Sleper and I am named as a co-inventor in this application. I am employed by the University of Missouri in Columbia, Missouri.
3. Exhibit A attached to this Declaration is my curriculum vitae summarizing my educational background and work experience.
4. I have reviewed the present claims and the Office Actions dated June 17, 2005, February 28, 2006 and October 18, 2006.
5. Exhibit B attached to this Declaration is a paper by Qiu et al. (Theor. Appl. Genet (1999) 98: 356-64). I was a co-author of this paper. This paper reports results from a study designed (1) to identify DNA markers that are linked to soybean cyst

nematode (SCN) resistance and (2) to identify DNA markers that are associated with seed protein and oil concentrations. While some DNA markers (e.g., B072) were found to be linked to genetic loci controlling both SCN resistance and seed protein or oil concentrations, other DNA markers (e.g., T005) were only associated with loci controlling SCN resistance, yet other DNA markers (e.g., B148) were only associated with loci controlling seed protein or oil concentrations. The conclusion of this paper is that these various DNA markers may be used to help select for strains with desirable traits such as SCN resistant and high seed protein concentration. However, this paper does not describe or suggest that a soybean strain's seed protein or oil concentration may be relied upon to predict the SCN susceptibility of the strain. This is because traits such as SCN resistance and seed protein or oil concentration may be controlled by more than one gene. See e.g., page 362, column 2, lines 21-24 of this paper. The paper does not show that IR spectra associated with seed protein or oil concentration is linked to SCN resistance.

6. The present application discloses a new methodology that uses spectra readings from soybean seeds to predict SCN susceptibility based on a predictive model that is constructed based on similar spectra readings from soybean strains with known or measurable SCN susceptibility. At the time of the present invention, no methodology was available to predict SCN resistance based on seed protein or oil content. Qiu et al. does suggest the use of DNA markers to help select for strains that are SCN resistant, but Qiu et al. never come close to suggesting that protein or oil content can be used to select for SCN resistant strains.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 1-22-07

By: 

David A. Sleper, Ph.D.

Professional Resume

Name: David A. Sleper
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Educational Experiences:

School	Location	Degree
Waldorf Jr. College	Forest City, IA	None
Iowa State University	Ames, IA	B.S., May 1967, (Agronomy)
Iowa State University	Ames, IA	M.S., August 1969, (Plant Breeding)
University of Wisconsin	Madison, WI	Ph.D., 1973, (Plant Breeding and Genetics)

Employment Record:

Position	Organization	Dates
Assistant Professor	University of Florida	1973-1974
Assistant Professor of Agronomy	University of Missouri;	1974-1978
Associate Professor of Agronomy	University of Missouri;	1978-1984
Professor of Agronomy	University of Missouri;	1984-Present
Shared Faculty with Cooperative States Research Service, Washington, D.C.		1985-1991
Coordinator of Agronomy and Horticulture	University of Missouri;	1991-97
Coordinator of Agronomy	University of Missouri	1997-99
Associate Director National Center For Soybean Biotechnology	University of Missouri	2005

Teaching Accomplishments:

One or more undergraduate and Graduate plant breeding courses taught since 1973
 Served as major professor to over 30 graduate students since 1974

Post-Doctorates:

C.F. Crane, 1979-83.

W.W. Xu, 1992.
M.M. Magai, 1992.
Mingshu Cao, 2001-04.
G. Lee 2004-05.
B. Gou 2005.

Visiting Scientists:

A) A. J. Harris, DSIR, Grassland Division
New Zealand, 1980-81.

B) R. Kopyto, Institute of Plant Breeding and Acclimatization,
Dept. of Fodder Crops, Krakow, Poland, 1983-84.

C) Levi S.M. Akundabweni, Department of Crop Science, University of Nairobi, Kenya, 1992.

D) J.Alberto Oliveira, C.I.A., La Coruña, Spain, 1995-96.

Refereed Publications since 2005 (over 140 total):

- Shannon, J.G., S.C. Anand, P.R. Arelli, J.A. Wrather, and D.A. Sleper. 2005. Registration of S99-3181 soybean. *Crop Sci.* 45: 407-408.
- MacDonald, R.S., J. Y. Guo, J. Copeland, S. Cole, J.D. Browning, Jr., D. Sleper, R. Kiengatti, G. Rottinghaus and M. Berhow. 2005. Environmental influences on isoflavones and saponins in soybeans and their role in colon cancer. *The Journal of Nutrition* 135:1239-1242.
- Shannon, J.G., D.A. Sleper, P.R. Arelli, J.W. Burton, R.F. Wilson, and S.C. Anand. 2005. Registration of S01-9269 Soybean germplasm line resistant to soybean cyst nematode with seed oil low in saturates. *Crop Sci.* 45: 1673-1674.
- Guo, B., D. A. Sleper, P. R. Arelli, J. G. Shannon, and H. T. Nguyen. 2005. Identification of QTLs associated with resistance to soybean cyst nematode races 2, 3 and 5 in soybean PI 90763. *Theor. Appl. Genet.* 111:965-971.
- Bilyeu, K, L. Palavalli, D. Sleper, and P. Beuselinck. 2005. Mutations in soybean microsomal omega-3 fatty acid desaturase genes reduce linolenic acid concentration in soybean seeds. *Crop Sci.* 45:1830-1836.
- Lu, P., J.G. Shannon, D.A. Sleper, H.T. Nguyen, S.R. Cianzo, and P.R. Arelli. 2006. Genetics of cyst nematode resistance in soybean PIs 467312 and 507354. *Euphytica* 149:259-265.
- B. Guo, D. A. Sleper, J. Sun, H. T. Nguyen, P.R. Arelli and J.G. Shannon. 2006. Pooled analysis of data from multiple quantitative trait locus mapping populations. *Theoretical and Applied Genetics* 113:39-48.
- Guo, B., D.A. Sleper, H.T. Nguyen, P.R. Arelli, and J.G. Shannon. 2006. Quantitative trait loci underlying resistance to three soybean cyst nematode populations in soybean PI 404198A. *Crop Sci.* 46:224-233.
- Guo, B., D.A. Sleper, P. Lu, J.G. Shannon, H.T. Nguyen, P.R. Arelli. 2006. QTLs Associated with Resistance to Soybean Cyst Nematode in Soybean: Meta-analysis of QTL locations. *Crop Sci.* 46:595-602.
- Beuselinck, P. R., D.A. Sleper, and K.D. Bilyeu. 2006. An Assessment of Phenotype Selection for Linolenic Acid Using Genetic Markers *Crop Sci.* 46: 747-750.
- Oliva, M.L., J.G. Shannon, D.A. Sleper, M.R. Ellersieck, A.J. Cardinal, R.L. Paris, and J.D. Lee. 2006. Stability of fatty acid profile in soybean genotypes with modified seed oil composition. *Crop Sci.* 46:2069-2075.

Bilyeu, K., L. Palavalli, D.A. Sleper, and P. Beuselinck. 2006. Molecular genetic resources for development of 1% linolenic acid soybeans. *Crop Sci.* 46:1913-1918.

Book:

Sleper, D.A. and J.M. Poehlman. 2006. *Breeding Field Crops*. 5th edition. 424 p. Blackwell Publishing. Ames, IA.

Professional Activities:

- 1) American Society of Agronomy
- 2) Crop Science Society of America
- 3) Genetics area program, University of Missouri
- 4) Graduate faculty, University of Missouri
- 5) Doctoral faculty, University of Missouri
- 6) Gamma Sigma Delta

Synergistic Activities:

My lab is involved in the breeding and genetics of soybean. Research emphasis is placed on developing cultivars, germplasm and breeding methodologies to screen for resistance to the soybean cyst nematode. Considerable emphasis is placed on mapping QTLs that are associated with resistance to the soybean cyst nematode. We have evaluated genotype x environmental interactions for isoflavone and saponin concentrations in elite and wild germplasm of soybean. We have developed many breeding lines and cultivars that are resistant to the soybean cyst nematode. Some of these lines have resistance to *Phytophthora* root rot as well. We have developed experimental soybean selections containing various modifications of fatty acids. We have developed experimental soybean selections of tofu, natto, and high protein.

- Served as President of the Crop Science Society of America in 2000. The Crop Science Society of America is a scientific organization with approximately 4,000 members from over 100 countries.
- Served as a consultant to Forage Genetics International, Inc. and to the Noble Foundation on issues associated with plant breeding.
- Coauthor of an undergraduate text in plant breeding entitled, "Breeding Field Crops". This is the most successful undergraduate text in the world on plant breeding.
- President of the American Society of Agronomy in 2005-06. ASA has over 11,000 members from over 100 countries.

B. X. Qiu · P. R. Arelli · D. A. Sleper

RFLP markers associated with soybean cyst nematode resistance and seed composition in a 'Peking' × 'Essex' population

Received: 3 March 1998 / Accepted: 18 August 1998

Abstract Soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe, causes severe damage to soybean [*Glycine max* (L.) Merr] throughout North America and worldwide. Molecular markers associated with loci conferring SCN resistance would be useful in breeding programs using marker-assisted selection (MAS). In this study, 200 F_{2:3} families derived from two contrasting parents, SCN-resistant 'Peking' with relatively low protein and oil concentrations, and SCN-susceptible 'Essex' with high protein and oil concentrations, were used to determine loci underlying the SCN resistance and seed composition. Three different SCN Race isolates (1, 3, and 5) were used to screen both parents and F_{2:3} families. The parents were surveyed with 216 restriction fragment length polymorphism (RFLP) probes with five different restriction enzymes. Fifty-six were polymorphic and contrasted with trait data from bioassays to identify molecular markers associated with loci controlling resistance to SCN and seed composition. Five RFLP markers, A593 and T005 on linkage group (LG) B, A018 on LG E, and K014 and B072 on LG H, were significantly linked to resistance loci for Race 1 isolate, which jointly explained 57.7% of the total phenotypic variation. Three markers (B072 and K014, both on LG H; T005 on LG B) were associated with resistance to the Race 3 isolate and jointly explained 21.4% of the total phenotypic variation. Two markers (K011 on LG I, A963 on LG E) associated with resistance to the Race 5 isolate together explained 14.0% of the total phenotypic variation. In the same

population we also identified two RFLP markers (B072 on LG H, B148 on LG F) associated with loci conferring protein concentration, which jointly explained 32.3% of the total phenotypic variation. Marker B072 was also linked to loci controlling the concentration of seed oil, which explained 21% of the total phenotypic variation. Clustering among quantitative trait loci (QTLs) conditioning resistance to different SCN Race isolates and seed protein and oil concentrations may exist in this population. We believe that markers located near these QTLs could be used to select for new SCN resistance and higher levels of seed protein and oil concentrations in breeding improved soybean cultivars.

Key words SCN · RFLP · QTL · Molecular marker · Soybean

Introduction

Soybean cyst nematode (SCN) is the most widespread pest infesting soybean plants. First reported in the United States in 1954 (Winstead et al. 1955), the SCN has since spread throughout most of the soybean production states. In 1988, the SCN was ranked as the number one crop disease in the southern United States (Sciubato 1993). The estimated reduction in soybean yield in the United States in 1994 was 1.99×10^6 tons (Wrather et al. 1995, 1997), amounting to approximately \$438.8 million loss to soybean producers. After field infestation with SCN, the number of nematodes may be reduced by carefully managing crop rotation, but it is difficult to completely eliminate them. The most efficient way to control the infestation of this pest is to plant SCN-resistant cultivars.

'Peking' is an important source of resistance to SCN, giving resistance to Races 1, 3, and 5. Genetic studies have shown that SCN resistance is conditioned by

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2000 (± 50) eggs were used to inoculate the roots of each 4-day-old seedling using an automatic pipet (Model 40A, Scientific Equipment, Baltimore, Md.) to ensure relatively accurate numbers of white females and cysts on all individual plants. Thirty days after inoculation, white females and cysts were dislodged from the roots using pressurized water, counted under a stereo-microscope, and then converted to index of parasitism (IP) values. The number of females and cysts from 5 individuals in each subgroup was averaged and used for evaluating the SCN response for its corresponding F_2 plant.

Based on the standard SCN classification systems (Golden et al. 1970; Riggs and Schmitt 1988) and previous genetic studies (Rao Arelli et al. 1992), the index of parasitism (IP) was calculated as follows, and used as bioassay data for regression analysis and MAPMAKER/QTL interval mapping.

$$IP = \frac{\text{Mean number of females in a given subfamily}}{\text{Mean number of females on susceptible 'Essex'}} \times 100$$

Seed protein and oil evaluation

The same $F_{2:3}$ families were used for seed protein and oil evaluation. These $F_{2:3}$ families were grown at the Bradford Research Center, University of Missouri, at Columbia in 1996. Twenty-five grams of dry seed from each of the $F_{2:3}$ families was used for measuring the percentage of protein and oil. These analyses were performed using a near-infrared (NIR) food and feed analyzer located at the USDA National Center for Agricultural Utilization Research, Peoria, Ill. All individuals with black seed-coat color were re-analyzed by grinding seeds into powder to avoid the misreading of extreme data by NIR due to the dark pigmentation. The original data of the seed protein and oil concentrations from each of the $F_{2:3}$ families were used for statistical analysis and QTL detection.

DNA preparation and hybridization

Standard methods were used for DNA preparation (Saghai Maroof et al. 1984). Briefly, young leaf tissues were harvested from the two parents and each of the 200 F_2 plants and used for DNA extraction and marker identification. Two-hundred and sixteen RFLP probes were used for screening both parents. The desirable clone/enzyme combinations from the screenings were selected for Southern hybridization in the 200 F_2 populations. DNA probes used in this study were initially developed by P. Keim and R. C. Shoemaker (Iowa State University, USDA-ARS, Ames, Iowa), and purchased from Biogenetic Services, Brookings, S.D. These inserts were recovered either by polymerase chain reaction (PCR) amplification (Saiki et al. 1988) or by stab-culturing and plasmid mini-preps. All DNA sam-

ples extracted from each of the 200 F_2 individuals were digested using five different restriction enzymes, *EcoRI*, *EcoRV*, *HindIII*, *DraI*, and *TaqI*. Digested DNA fragments were separated by 10 g/l (1.0%) agarose gel electrophoresis and transferred onto MSI magnacharge Nylon membrane (Micro Separations, Westborough, Mass.) using the method adopted by Southern (1975). Procedures for oligo-labelling were based on the methods of Feinberg and Vogelstein (1983).

Data analysis

Two-way regression analyses (SAS 1990) were performed to detect the association between restriction fragment length polymorphism (RFLP) markers and loci controlling resistance to SCN Race isolates, and QTL conferring protein and oil concentrations. Coefficient of regression (R^2) values were used for estimating the amount of phenotypic variation explained by the associated molecular markers.

Computer software MAPMAKER/EXP 3.0 (Lincoln et al. 1993) was used for determining genetic linkage and distance between different DNA markers by using maximum-likelihood analysis of the segregation for the RFLP-marker patterns in the F_2 populations. MAPMAKER/QTL 1.1 (Lincoln and Lander 1990) was used for scanning the QTLs in each linkage group of the genome. A LOD (logarithm of odd) score of 3.0 was set as the threshold in declaring linkage between a marker and a QTL. The position of the QTL relative to its nearby marker(s) was estimated based on the peaks from MAPMAKER/QTL scans.

Results

Distribution of SCN scores and seed compositions

The mean IP values of the $F_{2:3}$ families for Races 1, 3, and 5 isolates were 31.9, 41.8, and 32.7, respectively (Table 1). Based on the untransformed data, the distributions of Races 1, 3, and 5 were abnormal (Race 1: $W = 0.9449$, $P = 0.0001$; Race 3: $W = 0.9346$, $P = 0.0001$; Race 5: $W = 0.8694$, $P = 0.001$), with a slight shifting to the low cyst frequency side (Figs. 1A-C). After square-root transformation of the SCN score data, Races 1 and 3 showed a normal distribution with $W = 0.9786$, $P = 0.2463$ and $W = 0.9827$, $P = 0.6819$, respectively. However, the transformation of the SCN

Table 1 Mean and standard deviation (SD) of SCN scores and soybean protein and oil concentrations in parents and their $F_{2:3}$ individuals

SCN races and seed compositions	Peking		Essex		$F_{2:3}$	
	Mean ^a	SD	Mean ^a	SD	Mean ^a	SD
<i>Races^b</i>						
Race 1	0.60	0.89	308.25	27.02	31.88	38.90
Race 3	0.25	0.50	117.27	25.96	41.80	39.82
Race 5	1.00	1.00	201.50	20.51	32.74	25.47
<i>Seed composition^c</i>						
Protein (g/kg)	405.67	2.3785	425.67	2.3725	417.35	3.7865
Oil (g/kg)	164.67	2.0946	204.77	1.9556	186.36	3.0577

^{a,b} Mean IP values of SCN scores for Race isolates

^{a,c} Mean seed composition,

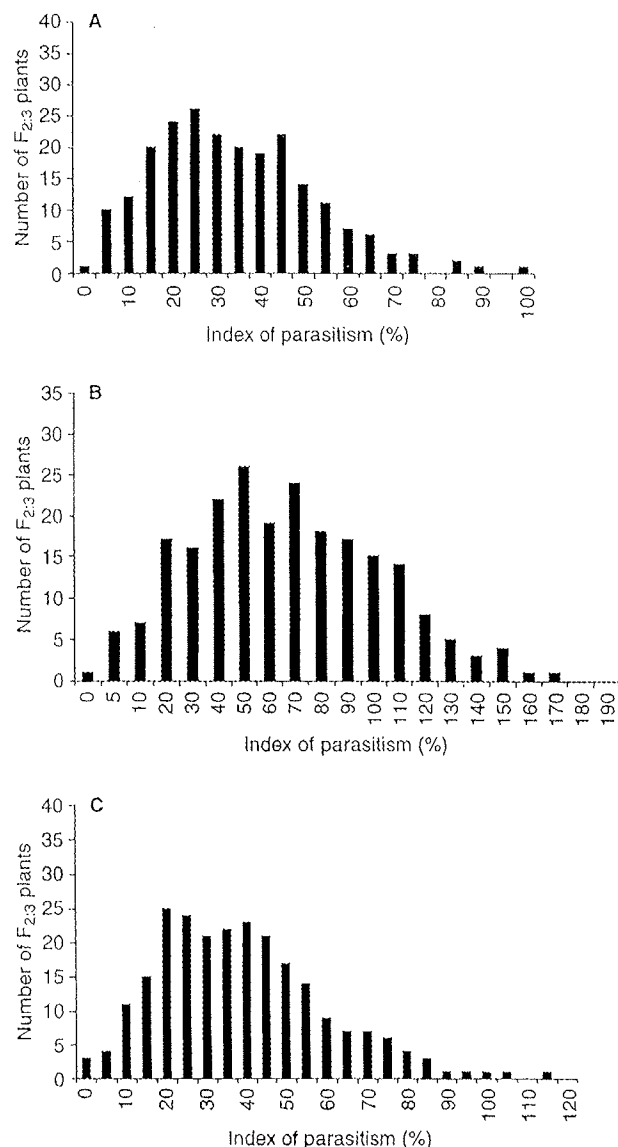


Fig. 1 **A** Frequency of distribution of the index of parasitism (IP) for SCN Race 1 among 200 $F_{2:3}$ families. The mean SCN score for 'Peking' was 0.60 (IP = 0.19) and for 'Essex' 79.00 (IP = 100). **B** Frequency of distribution of the index of parasitism (IP) for SCN Race 3 among 200 $F_{2:3}$ families. The mean SCN score for 'Peking' was 0.25 (IP = 0.22) and for 'Essex' 308.25 (IP = 100). **C** Frequency of distribution of the index of parasitism (IP) for SCN Race 5 among 200 $F_{2:3}$ families. The mean SCN score for 'Peking' was 1.00 (IP = 0.43) and for 'Essex' 246.00 (IP = 100)

score data for Race 5 did not achieve the normalization (Fig. 1C), with significant skewness (-0.3529) and kurtosis (-0.7932) remaining and $W = 0.9387$, $P = 0.0020$.

The protein concentration data showed an approximately normal distribution (Fig. 2A), with $W = 0.9675$ and $P = 0.0898$. The frequency distribution of the original data from oil concentration was not normal with significant skewness (1.3736) and kurtosis (5.6614), $W = 0.9314$ and $P = 0.0001$ (Fig. 2B), but the square-

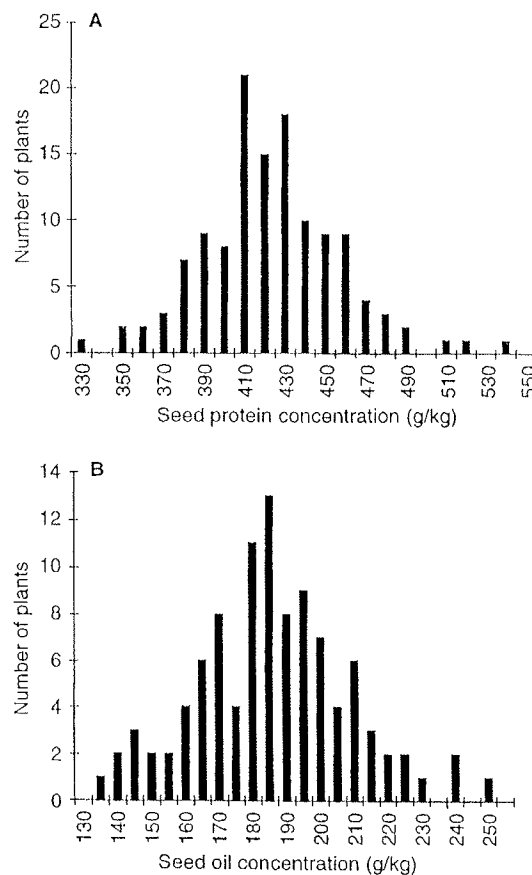


Fig. 2 **A** Frequency of distribution of seed protein concentration among $F_{2:3}$ families. The mean seed protein concentration for 'Peking' was 405.67 g/kg and for 'Essex' 425.67 g/kg. **B** Frequency of distribution of seed oil concentration among $F_{2:3}$ families. The mean seed oil concentration for 'Peking' was 164.67 g/kg and for 'Essex' 194.00 g/kg.

root transformation normalized the original data, with $W = 0.9621$ and $P = 0.0329$.

RFLP linkage map

A total of 216 RFLP probes were screened, and 56 were found to be polymorphic between parental lines. These polymorphic probes were further surveyed among the 200 F_2 individuals. The computer program MAPMAKER/QTL was used for anchoring the polymorphic markers, first then the anchored RFLP markers were used to identify the possible linkage groups based on information provided by the public soybean RFLP linkage map (Shoemaker and Specht 1995). Since there were only limited polymorphic markers in this study, the linkage map was generated tentatively, and only markers that were significantly associated with resistance to SCN or seed composition are presented (Table 2 and Fig. 3).

Table 2 Molecular markers significantly ($P < 0.01$) associated with resistance loci to SCN Race 1, 3 and 5, isolates and seed composition

Loci	L.G. ^a	R ²	P > F	Sources ^b	D/A ratio	Gene actions	Allelic means ^c		
							A	H	B
<i>Race 1</i>									
A593	B	0.21	0.0001	Peking	− 0.12	Additive	19	30	44
A018	E	0.16	0.0001	Peking	—	—	(25) ^d		
T005	B	0.15	0.0001	Peking	− 0.10	Additive	19	28	39
B072	H	0.13	0.0022	Peking	0.07	Additive	22	29	35
K014	H	0.12	0.0023	Peking	− 0.14	Additive	13	19	27
<i>Race 3</i>									
B072	H	0.13	0.0030	Peking	− 0.65	Partial Dominance	41	47	75
K014	H	0.09	0.0030	Peking	− 0.21	Partial Dominance	40	51	68
T005	B	0.09	0.0009	Peking	− 0.09	Additive	43	53	65
<i>Race 5</i>									
K011	I	0.11	0.0010	Peking	− 0.77	Partial Dominance	25	30	70
A963	E	0.09	0.0062	Peking	—	—	(33) ^d		
<i>Protein</i>									
B072	H	0.32	0.0018	Essex	0.20	Additive	46	43	41
B148	F	0.17	0.0001	Essex	0.50	Partial Dominance	45	42	41
<i>Oil</i>									
B072	H	0.21	0.0020	Essex	0.00	Additive	17	18	19

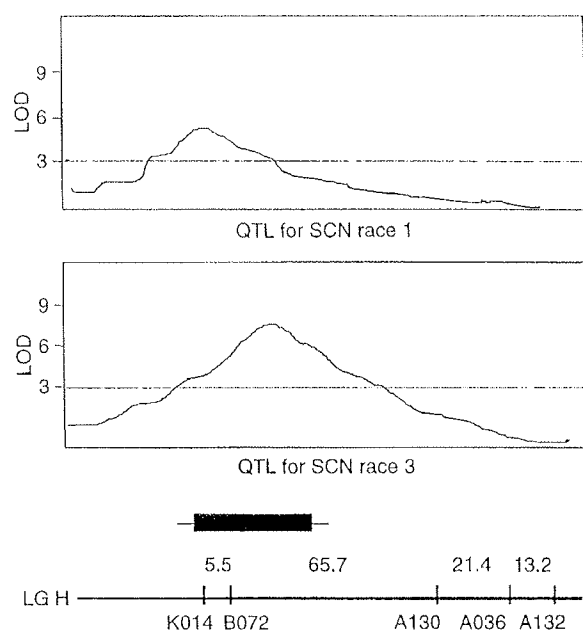
^a Soybean genome linkage group^b Sources of favorable alleles^c A, Homozygous alleles for resistance to SCN and higher level of seed protein concentration or lower level of oil concentration, H, heterozygous alleles for SCN resistance or seed composition, B, homozygous alleles for susceptibility to SCN and lower level of seed protein concentration or higher level of oil concentration^d Including both heterozygous and homozygous alleles for resistance to SCN, and seed composition

Fig. 3 MAPMAKER-QTL scans of the genomic regions on LG H covering SCN resistance loci for Races 1 and 3 isolates in a 'Peking' × 'Essex' population. Filled rectangle represents putative QTLs conferring SCN resistance to Races 1 and 3 isolates. Distances are given as Haldane centiMorgans (cM)

RFLP markers associated with SCN resistance

Associations between DNA markers and SCN resistance or seed composition were evaluated using both original and square-root transformed data in ANOVA and MAPMAKER/QTL analyses. Since the calculated R^2 values between the original and transformed data (data not shown) were not changed substantially, all results of the associations between DNA markers and traits using untransformed data are presented.

Five RFLP markers (A593, A018, T005, K014, and B072) were significantly associated with resistance to the SCN Race 1 isolate. Together they explained 57.7% of the total phenotypic variation (Table 3). Three markers (B072, K014, and T005) were significantly linked to resistance for Race 3 isolate (Table 2), jointly explaining 21.4% of the total phenotypic variation. Two markers (K011, and A963) were associated with resistance to SCN Race 5 isolate (Table 2), accounting for 14.0% of the total phenotypic variation. In this population it was found that all favorable alleles of resistance to SCN Races 1, 3, and 5 came from 'Peking' (Table 2), suggesting that 'Peking' is the donor of the resistance source.

On linkage group H, MAPMAKER-QTL scans showed one LOD peak near marker B072 for the QTL

Table 3 Combinations of RFLP markers and stepwise regression analysis for selected F_2 individuals for 'Peking' and 'Essex' markertypes against SCN Race 1 isolate

Combinations of markers ^a					<i>F</i> -test		
A593	A018	T005	B072	K014	<i>F</i>	<i>P</i> > <i>F</i>	<i>R</i> ²
+	+	—	—	—	19.06	0.0001**	0.2828
+	—	+	—	—	20.77	0.0001**	0.2956
+	—	—	+	—	12.27	0.0001**	0.3615
+	—	—	—	+	13.66	0.0001**	0.3664
—	+	+	—	—	16.29	0.0001**	0.2263
—	+	—	+	—	10.21	0.0001**	0.1755
—	+	—	—	+	5.69	0.0015*	0.1269
—	—	+	+	—	14.20	0.0001**	0.2831
—	—	+	—	+	10.93	0.0001**	0.2492
—	—	—	+	+	3.99	0.0110	0.1393
+	+	+	—	—	14.15	0.0001**	0.3742
+	+	—	+	—	8.38	0.0001**	0.4128
+	+	—	—	+	10.89	0.0001**	0.3764
+	—	+	+	—	8.99	0.0001**	0.4292
+	—	+	—	+	13.02	0.0001**	0.4213
+	—	—	+	+	11.13	0.0001**	0.3688
—	+	+	+	—	9.41	0.0001**	0.2758
—	+	+	—	+	8.54	0.0001**	0.2403
—	+	—	+	+	3.55	0.0042*	0.1353
—	—	+	+	+	5.73	0.0001**	0.2679
+	+	+	+	—	7.93	0.0001**	0.4569
+	+	+	—	+	8.78	0.0001**	0.6815
+	+	—	+	+	5.12	0.0001**	0.3789
+	—	+	+	+	9.18	0.0001**	0.5813
—	+	+	+	+	3.88	0.0005**	0.2585
+	+	+	+	+	6.80	0.0001**	0.5770

*Significant at $P < 0.005$; **Significant at $P < 0.005$

^a '+', present; '—', absent

conditioning resistance to Race 1 isolate (LOD = 5.0), and another peak on the other side of the same marker (LOD = 7.5) conditioning resistance to Race 3 isolate (Fig. 3). This indicated the locations of putative QTLs for resistance to SCN Race 1 and 3 isolates in 'Peking'.

Molecular markers that were associated with resistance to Race 1 showed additive gene action (Table 2). RFLP markers linked to Races 1 and 2 showed mostly partial dominance gene action except marker T005, which showed additive gene action.

Associated RFLP markers for protein and oil concentrations

RFLP marker B072 (LG H) was found to be significantly linked to protein concentration ($R^2 = 32\%$, $P < 0.0018$). Marker B148 (LG F) was also associated with protein concentration with a $R^2 = 17\%$ and $P < 0.0001$ (Table 2). These two markers jointly explained 33% of the total phenotypic variation. Marker B072 (LG H) was shown to be linked to loci governing oil ($R^2 = 21\%$, $P = 0.0020$) and protein concentrations (Table 1).

In this study, soybean seed protein concentration was found to be inversely correlated with seed oil concentration, with a correlation coefficient $r = -0.8860$

($R^2 = 0.7850$, $P = 0.0001$). This finding is consistent with the results from a study by Lark et al. (1994).

Molecular markers B072 (LG H) and B148 (LG F) had additive and partial dominant gene actions, respectively, in determining protein concentration. The sources of the favorable alleles for protein and oil concentrations in this study are all from 'Essex' (Table 2), implying that 'Essex' could be a potential source for breeding high levels of seed protein and oil concentrations. Compared with the parents, some heterozygous individuals in the $F_{2:3}$ progenies had extreme phenotypes exceeding those of the parental values (Table 1). This transgressive segregation pattern suggests that both parents might have beneficial alleles at different loci controlling protein and oil concentrations, which could allow their progenies to be valuable sources of new germplasm in breeding cultivars with high protein and oil concentrations.

Discussion

Clustering of QTL

The clustering of QTLs has reported for a number of crops (Martin et al. 1993; Mansur et al. 1996;

McMullen et al. 1995; Chang et al. 1997). Clusters of QTLs may also exist in this population. We found that five RFLP markers, located in LG B, E and H, were associated with loci controlling resistance to the SCN Race 1 isolate (Table 2). Among these 5 markers, three (B072 and K014 on LG H, T005 on LG B) were also linked to loci conferring resistance to the Race 3 isolate but not to the Race 5 isolate. The marker B072 on LG H was associated with both resistance loci to SCN (Races 1 and 3 isolates) and QTLs governing protein and oil concentrations (Table 2). The genomic region in LG H may contain a cluster of unique, but closely adjacent QTLs conferring traits. The possibility of pleiotropism among loci for SCN resistance to different Races was not eliminated in this population. In fact, MAPMAKER/QTL scans showed that the locations of QTLs conferring SCN resistance to both Races 1 and 3 isolates traversed across marker B072 (Fig. 3), suggesting the possible existence of a pleiotropic effect of a single gene for resistance to both Races 1 and 3 isolates. Data from regression analysis between mean IP scores from Race 1 and Race 3 isolates supported both the hypotheses of pleiotropism and clustering of QTLs ($R^2 = 8.05\%$, $P = 0.0001$).

Genetic variation of SCN QTLs

Several DNA markers have been reported to be associated with SCN Race 3 isolate in LGs A, G, and M in different populations (Webb et al. 1995; Concibido et al. 1996), including populations involving cv 'Peking' (Mahalingam and Skorupska 1995) or Forrest (Chang et al. 1997), a 'Peking'-derived SCN-resistant cultivar. Our results showed that DNA markers linked to resistance loci for SCN Races 1 and 3 isolates were involved in LGs B, E, and H. The different locations for the SCN resistance QTLs we identified in this population may indicate the existence of unique resistance loci in our soybean lines.

The genetic variation among QTL for resistance to SCN may vary in different populations. Molecular marker A006 (LG B) was found to be highly linked to a locus conferring the SCN Race 3 inbred isolate ($R^2 = 91\%$, $P = 0.0001$) in a population 'Williams \times Hartwig' (Vierling et al. 1996). Concibido et al. (1996) showed that marker Bng122 (LG G) was significantly linked to resistance for SCN Race 3 ($R^2 = 28.1\%$, $LOD = 6.94$) in a population derived from 'Evans \times Peking'. However, these two markers (A006 and Bng122) were not polymorphic in our population when screened using five different enzymes, *EcoRI*, *EcoRV*, *HindIII*, *DraI*, and *TaqI*. Mahalingam and Skorupska (1995) reported that RFLP marker A136 on LG A2 ($R^2 = 12.5\%$, $P = 0.0001$) and pA635 ($R^2 = 8.0\%$, $P = 0.0001$) were significantly linked to resistance loci for SCN Race 3 using an $F_{2:3}$ population from a cross between 'Peking' and 'Essex'. How-

ever, these RFLP markers did not reach significant levels in our population. The resistant 'Peking' used in each population could be different since genetic diversity among different sources of 'Peking' has been reported (Skorupska et al. 1994). These variations could also result from environmental factors, such as SCN screening in the greenhouse or genetic variation among SCN populations.

Mansur et al. (1993) reported that RFLP marker K1 was linked to loci controlling oil concentration ($R^2 = 11\%$, $P = 0.02$), and that marker L48 was associated with loci underlying soybean protein concentration ($R^2 = 20\%$, $P = 0.004$) in a F_5 population derived from a cross between 'Minsoy' (Bernard et al. 1988) and 'Noir1'. We used a different population, derived from a cross between 'Peking' and 'Essex', and found two different RFLP markers on LG H (B072) and LG F (B148) which were associated with loci controlling seed protein concentration. Marker B072 was also associated with loci conditioning oil concentration (Table 2). Data showing different molecular markers in different populations may indicate the existence of several different QTLs controlling seed protein and oil traits. Genome or population specificity of QTLs for soybean seed protein and oil concentrations has also been reported in another study (Diers 1992; Lee et al. 1996).

Marker-assisted selection

In breeding improved SCN-resistant cultivars using marker-assisted selection, it is necessary to choose the best marker combinations. We estimated the amount of phenotypic variations explained by different marker combinations for the Race 1 isolate. The efficiency for selecting resistant genotypes was evaluated using various combinations of five selected molecular markers in this study (Table 3). The 5 markers associated with resistance to the SCN Race 1 isolate could appear in 26 different combinations. Of these 26 combinations 23 statistically reached the $P < 0.001$ level, and two combinations reached the $P < 0.005$ level in regression analysis. The R^2 values ranged from 12.7% to 36.6% in the various two-marker combinations, 13.5% to 42.9% in the three-marker combinations, and 25.9% to 68.2% in different four-marker combinations (Table 3), indicating that when different marker combinations are used, different efficiencies in marker-assisted selection will likely result. A two-marker combination (e.g. A593 and K014) selected a total of 38 individuals in terms of parental genotypes, 20 with 'Peking'-type RFLP patterns and 18 with 'Essex'-type patterns, which explained 36% of the total phenotypic variation in the SCN responses. When a three-marker combination (A593, T005, and B72) was used as a selection tool, a total of 37 parental types were selected, explaining 42% of the total phenotypic variation. With a

four-marker combination (A593, A018, T005 and K014), approximately 68% of the total phenotypic variation to SCN reactions was explained. When all five markers (A593, A018, T005, B072 and K014) were used as a marker-assisted selection tool, 57% of the total phenotypic variation was explained (Table 3). These data show that different marker combinations could have different efficiencies in marker-assisted selection. In theory, the more markers used the more reliable marker-assisted selection would be because various markers could simultaneously select desirable soybean genotypes based on all these different markers. This may eliminate the selection of false positives based on only one marker. In breeding practices, consideration has to be given to the number of markers employed and the efficiency of different marker combinations. The comparison of efficiencies of different marker combinations may provide valuable information on choosing desirable marker combinations for optimizing marker-assisted selection in soybean breeding programs.

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References

- Bernard RL, Juvick GA, Hartwig EE, Edwards CJ Jr (1988) Origins and pedigree of public soybean varieties in the United States and Canada. US Dep Agric Tech Bull No. 1748
- Burton JW (1987) Quantitative genetics: results relevant to soybean breeding. In: Wilcox JR (ed) Soybeans: improvement, production, and use. (Agronomy, series, vol. 16). American Society of Agronomy, Madison, Wis., pp 211–247
- Buss GR, Camper HM Jr, Roane CW (1988) Registration of 'Hutcheson' soybean. Crop Sci 28: 1024
- Caldwell BE, Brim CA, Ross JP (1960) Inheritance of resistance of soybeans to the cyst nematode, *Heterodera glycines*. Agron J 52: 635–636
- Chang SJC, Doubler TW, Kilo VY, Abu-Threideh J, Prabhu R, Freire V, Suttner R, Klein J, Schmidt ME, Gibson PT, Lightfoot DA (1997) Association of loci underlying field resistance to Soybean Sudden Death Syndrome (SDS) and Cyst Nematode (SCN) Race 3. Crop Sci 37: 965–971
- Concibido VC, Denny RL, Boutin SR, Hautea R, Orf JH, Young ND (1994) DNA marker analysis of loci underlying resistance to soybean cyst nematode (*Heterodera glycines* Ichinohe). Crop Sci 34: 240–246
- Concibido VC, Young ND, Lange DA, Denny RL, Danash D, Orf JH (1996) Targeted comparative genome analysis and qualitative mapping of a major partial-resistance gene to the soybean cyst nematode. Theor Appl Genet 93: 234–241
- Diers BW, Shoemaker R (1992) Restriction fragment length polymorphism analysis of soybean fatty acid content. J Am Oil Chem Soc 69: 1242–1244
- Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction fragments to high specific activity. Anal Biochem 132: 6–13
- Golden AM, Epps JM, Riggs RD, Duclos LA, Fox JA, Bernard RL (1970) Terminology and identity of infraspecific forms of the soybean cyst nematode (*Heterodera glycines*). Plant Dis Rep 54: 544–546
- Hartl L, Weiss H, Zeller FJ, Jahoor A (1993) Use of RFLP markers for the identification of alleles of the *Pm3* locus conferring powdery mildew resistance in wheat (*Triticum aestivum* L.). Theor Appl Genet 86: 959–963
- Hartwig EE, Epps JM (1978) Registration of Bedford soybean. Crop Sci 18: 915
- Hospital F, Moreau L, Lacoudre F, Charcosset A, Gallais A (1997) More on the efficiency of marker-assisted selection. Theor Appl Genet 95: 1181–1189
- Lande R, Thompson R (1990) Efficiency of marker-assisted selection in the improvement of quantitative traits. Genetics 124: 743–756
- Lark KG, Orf J, Mansur LM (1994) Epistatic expression of quantitative trait loci (QTL) in soybean [*Glycine max* (L.) Merr.] determined by QTL association with RFLP alleles. Theor Appl Genet 88: 486–489
- Lee SH, Bailey MA, Mian MAR, Carter Jr TE, Shipe ER, Ashley DA, Parrott WA, Hussey RS, Boerma HR (1996) RFLP loci associated with soybean seed protein and oil content across populations and locations. Theor Appl Genet 93: 649–657
- Lincoln SE, Lander ES (1990) MAPMAKER/QTL. Whitehead Institute of Biomedical Research, Cambridge, Mass
- Lincoln SE, Daly MJ, Lander ES (1993) MAPMAKER/EXP. Whitehead Institute of Biomedical Research, Cambridge, Mass.
- Mahalingam R, Skorupska HT (1995) DNA markers for resistance to *Heterodera glycines* I. Race 3 in soybean cultivar 'Peking'. Breeding Sci 45: 435–443
- Mansur LM, Lark KG, Kross H, Oliveira A (1993) Interval mapping of quantitative trait loci for reproductive, morphological, and seed traits of soybean (*Glycine max* L.). Theor Appl Genet 86: 907–913
- Mansur LM, Orf JH, Chase K, Jarvick T, Cregan PB, Lark KG (1996) Genetic mapping of agronomic traits using recombinant inbred lines. Crop Sci 36: 1327–1336
- Martin GB, Brommonschenkel SH, Chunwongse J, Frary A, Ganai MW, Spirey R, Wu T, Earle ED, Tanksley SD (1993) Map-based cloning of a protein kinase gene conferring resistance in tomato. Science 262: 1432–1435
- Matson AL, Williams LF (1965) Evidence of a fourth gene for resistance to the soybean cyst nematode. Crop Sci 5: 477
- McMullen MD, Louie R (1991) Identification of a gene for resistance to wheat streak mosaic virus in maize. Phytopathology 81: 624–627
- McMullen MD, Simcox KD (1995) Genomic organization of disease and insect resistance genes in maize. MPMI 8: 811–815
- Myers GO, Anand SC, Rao Arelli AP (1989) Resistance to *Heterodera glycines* in soybean PI 437654. In: Pascale AJ (ed.) Proc 4th World Soybean Res Conf. Buenos Aires, Argentina, pp 1230–1234
- Pineda O, Bonierbale MW, Plaisted RL (1992) Identification of RFLP markers linked to the *H1* gene conferring resistance to the potato cyst nematode *Globodera rostochiensis*. Genome 36: 152–156
- Qiu BX, Slepier DA, Rao Arelli AP (1997) Genetic and molecular characterization of resistance to *Heterodera glycines* Races 1, 3, and 5 in 'Peking'. Euphytica 96: 225–231
- Rao Arelli AP (1994) Inheritance of resistance to *Heterodera glycines* Race 3 in soybean accessions. Plant Dis 78: 898–900
- Rao Arelli AP, Wrather JA, Anand SC (1992) Soybean resistance to soybean cyst nematode Race 3 is conditioned by an additional dominant gene. Crop Sci 32: 862–864
- Rao Arelli AP, Wilcox JA, Myers O Jr, Gibson PT (1997) Soybean germplasm resistant to Races 1 and 2 of *Heterodera glycines*. Crop Sci 37: 1367–1369

- Riggs RD, Schmitt DP (1988) Complete characterization of the Race scheme for *Heterodera glycines*. *J Nematol* 20:392–395
- Saghai Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proc Natl Acad Sci USA* 81:8014–8018
- Saiki RK, Gelfand DH, Stoffel S, Scharf S, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239:487–491
- SAS Institute (1990) SAS/STAT user's guide, version 6.0, 3rd edn. SAS Institute, Cary, N.C.
- Sciombato GL (1993) Soybean disease loss estimates for the southern United States during 1988–1991. *Plant Dis* 77:954–956
- Shoemaker RC, Specht JE (1995) Integration of the soybean molecular and classical genetic linkage groups. *Crop Sci* 35:436–446
- Skorupska HT, Choi IS, Rao Arelli AP, Bridges WC (1994) Resistance to soybean cyst nematode and molecular polymorphism in various sources of 'Peking' soybean. *Euphytica* 75:63–70
- Southern E (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 98:503–517
- Vierling RA, Faghihi J, Ferris VR, Ferris JM (1996) Association of RFLP markers with loci conferring broad-based resistance to the soybean cyst nematode (*Heterodera glycines*). *Theor Appl Genet* 92:83–86
- Webb DM, Baltazar BM, Rao Arelli AP, Schupp J, Clayton K, Keim P, Beavis WD (1995) Genetic mapping of soybean cyst nematode Race 3 resistance loci in the soybean PI 437654. *Theor Appl Genet* 91:574–581
- Winstead WW, Skotland CB, Sasser JW (1955) Soybean cyst nematode in North Carolina. *Plant Dis Rep* 39:9–11
- Wrather JA, Chambers AY, Fox JA, Moore WF, Sciombato GL (1995) Soybean disease loss estimates for the southern United States, 1974 to 1994. *Plant Dis* 79:1076–1079
- Wrather JA, Anderson TR, Arsyad DM, Gai J, Ploper LD, Portapuglia A, Ram HH, Yorinori JT (1997) Soybean Disease Loss Estimates for the Top 10 Soybean Producing Countries in 1994. *Plant Dis* 81:107–110
- Yu YG, Saghai Maroof MA, Buss GR (1996) Divergence and allelomorphic relationship of a soybean virus resistance gene based on tightly linked DNA microsatellite and RFLP markers. *Theor Appl Genet* 92:64–69